Report Prepared By: Dr. Britton Chance

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Contract: N-onr-23600

Annual Rate: $8,415 (plus an $860 supplement from the University of Pennsylvania)

Contractor: The University of Pennsylvania

Principal Investigator: Dr. Britton Chance

Assistants: Dr. C. C. Yang, January 1 - July 1, 1953
Dr. Joseph E. Hayes, Jr., July 15 - December 24, 1953
Henry Altschuller, July 1, 1952 - January 30, 1954
Louis Ferretti, October 28, 1953 - January 13, 1954
Joseph Higgins, January 1, 1953 - January 13, 1954

Title of Project: "The Properties of Enzymatic Systems: A Theoretical and Experimental Study"

OBJECTIVES: An experimental study of the nature of labile intermediates in enzyme action combined with the theoretical study of how such intermediates should act according to various theories of physical chemistry.

ABSTRACT OF RESULTS:

a. Results since start of project: The availability of our electric analog computer has permitted us to elucidate in detail the reaction kinetics of the catalase system, and to verify in some detail our mechanism for its action. It has permitted us to elucidate certain peculiarities in the reaction of peroxidase in the presence of oxidase systems that produce hydrogen peroxide (coupled oxidations).

We have proposed a collision hypothesis for the mechanism of action of insoluble enzymes that are tightly bound to particles of muscle or liver, but which may react by limited movements leading to collisions of adjacent enzymes. It has further been possible to set up some general theorems concerning the kinetics of oxidation and reduction of the components of a sequence of enzyme reactions. The consequences of this hypothesis have been examined by means of the computer and a practical application to the cytochrome components of the succinic oxidase system has been possible.

b. Progress during the current report period: Dehydrogenase Systems. Studies of the kinetics of an intermediate compound of glyceraldehyde-3-phosphate dehydrogenase and diphosphopyridine nucleotide (DPN) opened up a new area of spectrophotometric investigation of reaction kinetics in dehydrogenase systems. The experimental work which was carried on in collaboration with Dr. J. Harting was published in

abstract form and presented at the 1953 Federation meeting. Since that time, the investigations have been extended to the study of this compound in respiring yeast cells. Such a compound has been detected there and has provided a direct assay of the amount of DPN bound to this glyceraldehyde phosphate dehydrogenase. Results of this study were reported at the McCollum-Pratt symposium on Enzyme Mechanisms and the full report is published in the volume entitled Enzyme Metabolism.

A further extension of our studies of DPN linked dehydrogenases was provided by the work that Dr. Joseph E. Hayes, Jr., did during his six-months stay at this laboratory. Whereas he had previously obtained from measurements of the overall reaction kinetics the velocity constants for the forward reaction with alcohol, his work in this laboratory with a rapid recording spectrophotometer permitted him to obtain preliminary values for the velocity constants of the reactions in the opposite direction leading to a complete determination of the kinetics of this system which are represented in the following diagram below.

I. $$E + DPNH \underset{100 \ sec^{-1}}{\overset{9\times10^{6} \ M^{-1} \ sec^{-1}}{\rightleftharpoons}} E\cdot DPNH$$

$$E\cdot DPNH + Ald \underset{(1\times10^{4} \ M^{-1} sec^{-1})}{\overset{3\times10^{6} \ M^{-1} \ sec^{-1}}{\rightleftharpoons}} E\cdot DPN + Alc$$

$$E\cdot DPN \underset{5\times10^{6} \ M^{-1} \ sec^{-1}}{\overset{600 \ sec^{-1}}{\rightleftharpoons}} E + DPN$$

$$\frac{k_{-1} \ k_{-2} \ k_{-3}}{k_{1} \ k_{2} \ k_{3}} = 3.4\times10^{-4} = K_{eq.} \qquad (1.0\times10^{-3} \text{ observed})$$

II. $$E + DPN \underset{200\ sec^{-1}}{\overset{2\times10^{6}\ M^{-1}\ sec^{-1}}{\rightleftharpoons}} E\cdot DPN$$

$$E\cdot DPN + Alc \underset{(3\times10^{6}\ M^{-1}\ sec^{-1})}{\overset{1\times10^{4}\ M^{-1}\ sec^{-1}}{\rightleftharpoons}} E\cdot DPNH + Ald$$

$$E\cdot DPNH \underset{2\times10^{7}\ M^{-1}\ sec^{-1}}{\overset{200\ sec^{-1}}{\rightleftharpoons}} E + DPNH$$

$$\frac{k_{-1}\ k_{-2}\ k_{-3}}{k_{1}\ k_{2}\ k_{3}} \doteq 2.9\times10^{-4} = K_{eq.} \qquad (1.0\times10^{-3}\ \text{observed})$$

From these data the equilibrium constant can be calculated and is found to be within a factor of three of that determined directly from the equilibrium. Such overall comparisons of kinetic and equilibrium data are of considerable value in establishing the validity of our studies.

On the theoretical aspects of dehydrogenase action, we have investigated several possible mechanisms for glyceraldehyde phosphate dehydrogenase. The analysis that we carried out was sufficiently incisive to tell us that there are serious discrepancies between the kinetics of reduction of DPN and the kinetics of formation and disappearance of the glyceraldehyde phosphate dehydrogenase-DPN complex as registered by our spectrophotometer. This means that there are unknown reaction steps that cannot be examined spectrophotometrically at the present time. It is our conclusion that this problem should be set aside until a more sensitive spectrophotometric method could be developed whereby a more accurate comparison of the time course of DPN reduction and the kinetics of the complex could be obtained.

Succinic Oxidase System: The experimental aspects of our kinetic and steady-state analysis of the succinic oxidase system have moved

forward again this fall. We have obtained more accurate and extensive data on the speed of reaction of succinate with the cytochrome b component of the cyanide-inhibited succinic oxidase system. Furthermore, we have been able to titrate succinate-reduced cytochrome b with fumarate and are able to give values for the ratio of fumarate-to-succinate that give equal ratios of reduced-to-oxidized cytochrome b. When calculated as oxidation-reduction potentials, values considerably different from those now accepted in the literature are obtained. Further work is being continued in order to discover the cause of such discrepancies.

On the theoretical side, we are setting up our computer to represent the kinetics of reduction of cytochrome by succinate, taking into account the presence of fumarate and the reversible reactions involved, in order to show whether cytochrome b reacts fast enough with succinate to engage in electron transport in the heart muscle preparation.

It is our hypothesis that cytochrome b has lost a portion of its physiological function because of the preparation method recommended by Keilin and Hartree, which, of course, leads to maximal succinic oxidase activity. It is interesting to speculate that one of the functions that cytochrome b has lost in its preparation is that of oxidative phosphorylation. Indirect support for this hypothesis is that in liver mitochondria where oxidative phosphorylation can occur, we find the kinetics of reduction of cytochrome b to agree much more closely with those of cytochrome c than in the heart muscle preparation, which, as mentioned above, has already lost the capacity for oxidative phosphorylation.

Oxidative Phosphorylation. Experimental work that is not supported by this contract but which furnishes most interesting data for analysis is our recent discovery of spectroscopic changes that accompany the initiation of oxidative phosphorylation in rat liver mitochondria. An analysis of the reaction kinetics that occur upon the addition of adenosine diphosphate to these mitochondria will be most interesting. It is our hope to be able to determine on a kinetic basis those respiratory enzymes which are involved in oxidative phosphorylation by measuring the speeds with which a change in a steady-state level occurs upon the addition of adenosine diphosphate.

Rapid kinetic studies of the speeds of oxidation of the reduced respiratory pigments of various materials. With the aid of a new form of the regenerative flow apparatus, it is now possible to measure the speeds of oxidation of cytochrome a_3, a, c, b, flavoprotein, and reduced pyridine nucleotide in suspensions of yeast cells, or in ascites tumor cells. In bacteria that contain various other types of respiratory pigments it is possible to study their kinetics as well. These data are of the greatest importance for our theoretical studies of the action of sequential enzyme systems. Our preliminary results show that oxidizing equivalents are carried rapidly up through cytochrome c; thereafter slower oxidations of cytochrome b, flavoprotein, and reduced pyridine nucleotide occur. Our current hypothesis is that the system of cytochrome a_3, a, and c is a closely knit one in which oxidizing equivalents can be rapidly transported through cytochrome c (and possibly through Slater's factor as well). Thereafter the system may branch off into several directions: i.e., toward cytochrome b, toward diaphorase, or toward other types of cytochrome c reductases. We would then suppose that

it is this branching of the oxidation chain that causes the reaction velocity to diminish.

Technical assistance. This contract has rendered valuable service to the work mentioned above in terms of technical assistance in the preparation of enzymes, in the culture of microorganisms, etc.

Technological improvements. This laboratory was fortunate enough to receive from the ONR (Office of Naval Research) funds for the improvement of the existing methods for the detection of small changes of optical density. In order to test certain theoretical relationships between the design variables that we had formulated, a new double-beam spectrophotometer especially designed for studies in the ultraviolet region of the spectrum is nearly completed. In the course of tests of the sensitivity of this apparatus it will also be possible to see whether or not some enzyme reactions that are barely detectable at present can be accurately recorded by this new apparatus. A problem on which we have been able to obtain results so far only with the greatest of difficulty is the detection of absorption in the neighborhood of 240 mμ which might be attributed to the formation of thiol esters between the dehydrogenases and their substrates. It is believed that the preliminary results that we obtained with glyceraldehyde-3-phosphate dehydrogenase and that were published in the Federation Proceedings by Harting and Chance could be considerably improved in accuracy and extended to other enzyme systems.

PLANS FOR THE FUTURE:

Immediate: Our immediate plans for the future are to prepare for publication our work on cytochrome b, on ascites tumor cells, and on the kinetics of oxidation of the reduced respiratory

pigments. This will involve considerable work with the analog computer in order to evaluate critically the theories by which we have proposed to explain the reaction mentioned previously.

We also plan to broaden the scope of our theoretical studies of enzyme systems, and to follow up some promising leads for analytical methods for the solution of equations representing enzyme kinetics. See, for example, C. C. Yang, "A Reversion Method for the Solution of Differential Equations Representing Enzyme Kinetics," submitted to Archives of Biochemistry and Biophysics.

Our most exciting project for the next year will, however, be a theoretical analysis of the kinetic data that we expect to obtain on the kinetics of initiation of phosphorylation in rat liver mitochondria.

Long Range: Our long range plans are to develop and to broaden the scope of our studies of the dynamics of intracellular enzyme systems. We have a unique opportunity to apply our powerful physical methods in combination with the unusual facilities for mathematical analysis of enzyme systems to the most interesting problem of enzyme action in intact cells.

BIBLIOGRAPHY:

Britton Chance, The Carbon Monoxide Compounds of the Cytochrome Oxidases.

I. Difference Spectra, Journal of Biological Chemistry, 202, 383 (1953).

II. Photodissociation Spectra, Journal of Biological Chemistry, 202, 397 (1953).

III. Molecular Extinction Coefficients, Journal of Biological Chemistry, 202, 407 (1953).

Britton Chance, Lucile Smith and La Roy Castor, New Methods for the Study of the Carbon Monoxide Compounds of Respiratory Enzymes, Biochemica et Biophysica Acta, 12, 289 (1953).

A contribution was made to the McCollum-Pratt Symposium on "Mechanism of Enzyme Action" and will be published in the book, "Enzyme Metabolism," early this year:

Britton Chance, Enzyme-Substrate Compounds in Living Cells.

An abstract has been submitted to the Society of Biological Chemists for presentation at the April meeting:

Britton Chance and G. R. Williams, The Steady-State of Reduced Pyridine Nucleotides in Rat Liver Mitochondria.

C. C. Yang, A Reversion Method for the Solution of Differential Equations Representing Enzyme Kinetics, submitted to Archives of Biochemistry and Biophysics.

Britton Chance and Jane Harting, Some reactions of Glyceraldehyde-3-phosphate dehydrogenase, Federation Proceedings, 12, 188 (1953).

Jane Harting and Britton Chance, Federation Proceedings, 12 (1953).